Both the Antioxidant and D₃ Agonist Actions of Pramipexole Mediate Its Neuroprotective Actions in Mesencephalic Cultures

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ABSTRACT

Pramipexole (PPX) is a full intrinsic activity, direct-acting dopamine (DA) agonist possessing 7-fold higher affinity for D₃ than for D₂ receptors. It also is a potent antioxidant. PPX was previously shown to be neuroprotective because it dose dependently attenuated the DA neuron loss produced by levodopa in mesencephalic cultures. Several different drugs with properties similar to PPX were studied here to better understand the mechanism or mechanisms responsible for this neuroprotective effect. The D₃-preferring agonist 7-hydroxy-diphenylaminotetralin (7-OH-DPAT) and the D₃ antagonist U99194, respectively, increased and decreased the neuroprotective effects of PPX in a dose-dependent fashion. Addition of the selective D₂ agonist U95666 or the D₂/D₃ antagonists domperidone or raclopride did not affect PPX’s neuroprotective effect. Interestingly, 7-OH-DPAT by itself did not attenuate the DA neuron loss produced by levodopa. However, when 7-OH-DPAT was combined with a low dose of the antioxidants U101033E or a-tocopherol, the toxic effects of levodopa were attenuated. Similar results were observed when the D₃-preferring agonist PD128,907 was studied. In addition, media conditioned by exposure of mesencephalic cultures incubated with all D₃-preferring agonists studied was shown to enhance the growth of DA neurons in freshly harvested recipient cultures implicating a D₃-mediated trophic activity in the neuroprotective effect. These data suggest that PPX’s neuroprotective actions in the levodopa toxicity model are a consequence of its combined actions as a D₃ receptor agonist and an antioxidant.

Pramipexole (PPX; Mirapex) is a direct-acting dopamine (DA) receptor agonist with full intrinsic activity at the D₂ receptor family of receptors (Piercey et al., 1995). Competitive binding studies reveal that PPX has a 7-fold higher affinity for the D₂ receptor subtype than for the D₃ or D₄ receptors (Piercey et al., 1996a). In low concentrations, PPX, presumably acting at DA autoreceptors, lowered the rate of DA synthesis, release, and firing, thereby reducing extracellular DA concentrations (Carter and Muller, 1991). When examined in vivo, low dosages of PPX reduced haloperidol-induced dyskinesias and locomotor activity (Mierau and Shingnitz, 1992), both of which are thought to result in part from increased DA activity. At higher concentrations, PPX also stimulated postsynaptic DA receptors and has been shown to reverse Parkinson-like symptoms in rats and monkeys exposed to DA neurotoxins (Domino et al., 1997). This action is likely responsible for its efficacy in patients with Parkinson’s disease (PD) (Shannon et al., 1997). In addition to its dopaminergic properties, PPX has antioxidant actions (Hall et al., 1996; Piercey, 1998; Thomas Hinds, University of Washington, personal communication). This antioxidant action is likely responsible for the ability of PPX to reduce levodopa toxicity in cerebellar cultures (Althaus et al., 1996). Its antioxidant actions, along with its ability to reduce DA stress thought to contribute to the neurodegenerative process underlying this disease (Olanow, 1992).

It is well established that both levodopa and DA are toxic to DA neurons in vitro, presumably through oxidant stress (Graham et al., 1978). Previously, our laboratory demonstrated that PPX dose dependently reduced levodopa-induced DA neuron loss in cultures, whereas other DA agonists, in

ABBREVIATIONS: CM, complete media; DA, dopamine; DM, defined media; DMEM, Dulbecco’s modified Eagle’s medium; MPP⁺, 1-methyl-4-phenylpyridinium; PC, plate control; 7-OH-DPAT, 7-hydroxy-diphenylaminotetralin; PD, Parkinson’s disease; PD128,907, (+)-trans-3,4,4a,10b-tetrahydro-4-propyl-2H,5H-(1)-benzopyrano-(4,3b)-1,4-oxasind-9-ol; PPX, pramipexole; RMT, rostral mesencephalic tegmentum; TH, tyrosine hydroxylase; THir, TH immunoreactive; U101033E, pyrrolopyrimidine lipid peroxidation inhibitor (lazeroid compound); U95666, (R)-5,6-dihydro-5-(methylamino)-4H-imidazo-(4,5,1-j)-quinolin-2(1H)-one monohydrochloride; U99194, 5,6-dimethoxy-N,N-dipropyl-2-indanamine monochloride.
including pergolide and bromocriptine, were without effect (Carvey et al., 1997a). Similarly, Zou et al. (1997) demonstrated the ability of PPX to attenuate levodopa-induced DA neuron loss in an immortalized mesencephalic cell line. Although these neuroprotective effects could be a consequence of the antioxidant actions of PPX, the fact that the effect was in part stereoselective argues for the involvement of additional mechanisms. Recent data suggest that one of these mechanisms may involve an increased production of a DA neuron-derived neurotrophic factor (Ling et al., 1998b).

Conditioned media from mesencephalic cultures transferred to freshly harvested mesencephalic cultures normally stimulates DA neuron growth in the recipient cultures. This conditioned media transfer effect was increased by incubating the donor cultures with PPX (Carvey and Ling, 1997a). The magnitude of this effect was dependent on the concentration of PPX in the donor cultures and was not observed when parietal cortex cultures were used as the donor source. Heat inactivation of the donor media from mesencephalic cultures dramatically reduced this effect, suggesting the involvement of a protein. If the donor cultures were pretreated with the select DA neurotoxin 1-methyl-4-phenylpyridinium (MPP+), the PPX-induced conditioned media transfer effect was totally abolished (Carvey and Ling, 1997a). In contrast, PPX was still able to enhance the trophic activity in cultures without glia (Ling et al., 1998b). A 35-kDa candidate protein was recently identified (Ling et al., 1998b). SDS-polyacrylamide gel electrophoresis revealed that this protein was present in normal cultures, increased by PPX, and abolished by MPP+ pretreatment. Taken together, these data suggest that PPX increases the production of a trophic protein normally produced by the DA neuron, although they do not rule out GABAergic neurons as the source of the trophic activity.

In addition to these in vitro studies, several in vivo studies support a neuroprotective role for PPX. Thus, we demonstrated that mesencephalic extracts, but not cerebellar extracts, from rats chronically treated with varying doses of PPX increased the survival of DA neurons in mesencephalic cultures (Carvey and Ling, 1997b). As was true of the in vitro studies, this trophic effect was dose dependent, heat labile, and not observed when the DA agonist pergolide was studied. PPX has also been shown to attenuate the dopaminergic toxic effects produced by high-dose methamphetamine (Hall et al., 1996) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Kitamura et al., 1997). These animal studies extend the in vitro findings and suggest that the neuroprotective effects of PPX in vivo may also involve an increased production of a neurotrophic factor. Unfortunately, we do not know whether the antioxidant effects of PPX, its DA agonist properties, or some unknown action of this drug is responsible for its neuroprotection. We have begun to address this issue.

In the present study, we evaluated several drugs possessing affinity for D2 and/or D3 receptor sites as well as drugs possessing antioxidant activity. By studying the effects of these drugs in mesencephalic cultures or in combination with levodopa (or with PPX and levodopa), we hoped to determine the relative contribution of the D2, D3, and antioxidant actions of PPX in its neuroprotective effect.

Materials and Methods

Drugs. PPX, the D3 antagonist U99194 (5,6-dimethoxy-N,N-dipropyl-2-indanamine monochloride), the D2 agonist U95666 (R)-5,6-dihydroxy-5-(methylamino)-4H-imidazo-(4,5,1-i-j)-quinolin-2(1H)-one monohydrochloride, and the pyrrolopyrimidine lipid peroxidation inhibitor (lazedor compound/antioxidant U101033E (9-[2-(4-morpholinoethyl)-2,4-di[1-pyridylidinyl]-6,7,8,9-tetrahydro-5H-pyrrolo[4,5-b]indole dihydrochloride salt) were all obtained from Pharmacia and Upjohn (Kalamazoo, MI). The D3 preferring agonists 7-hydroxy-diphenylaminotetralin (7-OH-DPAT) and PD128,907 [(1-t-trans-3,4,4a,10b-tetrahydro-4-propyl-2H,5H-(1)-benzpyrano-[3,4-b]-1,4-oxazin-9-0l) as well as the mixed D2/D3 agonists raclopride and domperidone were purchased from RBI (Natick, MA). α-Toxopheryl and levodopa were purchased from Sigma Chemical Co. (St. Louis, MO).

Rostral Mesencephalic Tegmentum Isolation. For each experiment, precisely timed gravid (E15) Sprague-Dawley rats (Zivic-Miller, Zenopolis, OH) were administered pentobarbital (60 mg/kg; The Butler Company, Columbus, OH) via i.m. injections, and the embryos were removed from the animal. Embryos were then placed in a chilled dissection solution consisting of Hanks’ balanced salt solution (GIBCO, Grand Island, NY), 1% penicillin-streptomycin (Pen-Strep; Sigma Chemical Co.), and 1% d-glucose (Sigma Chemical Co.). Using the method described by Carvey et al. (1997b), embryonic brains were removed, and the rostral mesencephalic tegmentum (RMT) was bilaterally dissected free from the brains and placed in dissection solution.

After removal, collected RMT tissue was enzymatically dissociated by first incubating the tissue in 0.1% trypsin (Sigma Chemical Co.) for 30 min at room temperature and then in a solution of 40 μg/ml deoxyribonuclease (DNase; Sigma Chemical Co.) and trypsin inhibitor (Sigma Chemical Co.) for 10 min at 37°C. Tissue was then placed into complete media (CM) containing 44.5% Dulbecco’s modified Eagle’s medium (DMEM; GIBCO), 44.5% F-12 Nutrient Mixture (GIBCO), 10% fetal calf serum (FCS; Sigma Chemical Co.), and 1% Pen-Strep and triturated into a single cell suspension by using a fire-polished pasteur pipette.

RMT Cell Cultures. After preparation, RMT cells were plated onto polyl-lysine (Sigma Chemical Co.) coated 48-well plates (Costar) at a density of 250,000 viable (trypan blue excluding) cells/cm². During plating, various drugs were added to the cultures to satisfy the experimental conditions. With the exception of the pyrrolopyrimidine antioxidant (U101033E) and α-tocopherol, all the drugs were dissolved in an Hanks’ balanced salt solution vehicle. The U101033E was dissolved in 1.0% Intralipid (Kabi Pharmacia, Clayton, NC), whereas α-tocopherol was dissolved in absolute alcohol. When α-tocopherol was added to cultures, 5 μl of this concentrate was added to a total of 500 μl media leaving the ethanol concentration in the cultures at 1%. In studies involving α-tocopherol, appropriate control cultures containing 1.0% ethanol were included. Control cultures for the U101033E studies similarly included 1.0% Intralipid. For each experiment, the cultures were incubated at 37°C in a 5% CO2 humidified incubator. After 72 h or 7 days of incubation (depending on the experimental paradigm), the RMT cultures were fixed at room temperature with 3.7% paraformaldehyde for a period of 30 min and subsequently stained for the DA neuron marker tyrosine hydroxylase (TH) as previously described (Ling et al., 1998a).

TH Staining. Cultures were incubated for 60 min with a solution of 0.25% Triton X-100 and 3% horse serum and then incubated with a monoclonal anti-TH primary antibody (1:5000; Incstar, Stillwater, MN) overnight at 4°C. Sequential 60-min incubations with horse anti-mouse secondary antibody (0.5%) and peroxidase conjugated avidin-biotin complex (ABC; Vector Laboratories, Minneapolis, MN) were performed. The TH immunoreactivity was developed with a chromogen containing 0.05% 3,3’-diaminobenzidine, 2.5% nickel sulfate, and 0.005% H2O2.

Cell Count Assessment. The number of TH immunoreactive (THir) cells was used as an index of DA neuron number in all cases. An investigator blinded to treatment history counted the number of THir figures in a swath (28% of surface area) down and across the center of each well using a Leitz Fluovert (Germany) reverse-phase
mircosof. This number was used in all subsequent statistical analyses. Each culture plate also contained two sets of control cultures. The “positive control” (two wells) contained CM only (as described above, which contained 10% FCS), whereas the “plate control” (PC; n = 2 wells) contained media consisting of 49.5% DMEM, 49.5% Hamm’s F-12, and 1% penicillin-streptomycin. In those studies where α-tocopherol or Intralipid was involved, additional control cultures (n = 2 each; 1% ethanol or Intralipid) were added to each plate as described above. For a study to be considered valid, the positive control THir cell counts had to be at least 1.75 times the PCs. If this criterion was not achieved, the cultures were discarded and the experiment was repeated.

**Experimental Paradigms.** Five series of experiments were performed. In all studies, each drug concentration was evaluated in two wells. The drugs were assessed in cultures incubated with CM containing 10% FCS unless otherwise stated. Each study was replicated at least once using a new batch of freshly harvested RMT cultures. In the first series, varying concentrations of each drug (100 pM to 100 μM) were added to the RMT cultures and incubated for 72 h, after which THir cell counts were assessed. These “survival” studies were performed to determine whether the drugs themselves were toxic or trophic to DA neurons. Results were standardized across all studies by expressing the values as percentage cell counts in CM only for that experiment. In the second series, the study drugs were added with 10 μM levodopa and PPX. These “combination” studies were used to determine whether any of the drugs could increase or decrease the attenuation of PPX of the THir cell loss normally produced by levodopa. In these experiments, two wells on each plate were incubated with just levodopa to ensure that toxicity was occurring. Because it was anticipated that 7-OH-DPAT and other agonists would increase survival, 100 nM PPX was used to avoid a ceiling effect. In contrast, 1 μM PPX was used for the antagonist studies to avoid a floor effect. In these studies, the results were standardized by expressing the values as percentage of cell counts in the levodopa plus PPX cultures. In the third series, the drugs were added at various concentrations (1 nM to 100 μM) along with a fixed concentration of levodopa (10 μM). These “neuroprotection” studies evaluated whether the drugs by themselves were able to attenuate levodopa-induced THir cell loss. In a fourth series of “antioxidant” studies, 7-OH-DPAT (1 μM) or PD128,907 (100 nM) and U101033E (1 μM) or α-tocopherol were added to RMT cultures containing various concentrations of levodopa. In a final series of “conditioned media transfer studies,” RMT cells were cultured in CM that was then replaced 24 h later by the study drugs containing defined media (DM) consisting of DMEM/Hamm’s F-12:1:1 Gibco; transferrin (100 μg/ml), insulin (25 μg/ml), progesterone (50 μM), putrescine (62 μM), sodium selenite (30 nM), penicillin-streptomycin, and glucose (33 mM). Supernatants (conditioned media) were collected after an additional 6-day incubation at 37°C and transferred to freshly harvested “recipient” RMT cultures growing in DM. After 72 h, the trophic activities in the conditioned media were assessed by the comparison of THir cell counts in conditioned media-added wells with those in control wells. In some of these experiments, the conditioned media was processed using centrifugal filtration units (Millipore Ultrafree-MC) to separate the media into fractions containing <10- or >10-kDa molecules. These fractions (50% v/v) were subsequently added to freshly harvested RMT cultures growing in DM and, after 72 h, assessed for THir cell counts.

**Statistical Analysis.** The THir cell counts were analyzed using one-way or multiway ANOVA (SPSS software, version 6.1). When ANOVA revealed a statistically significant effect of drug or dose, the individual treatment groups were analyzed by using Newman-Keuls post-hoc test (p < .05).

**Results**

**Survival studies.** All drugs were evaluated in RMT cultures incubated in CM to determine whether they altered THir cell counts. PPX (F = 35.74; p < .001) and 7-OH-DPAT (F = 15.89; p < .001) dose dependently increased THir cell counts in the cultures (Fig. 1). Thus, after 72 h, the THir cell counts were maximally increased by 98% and 64%, respectively, relative to control cultures incubated with CM only. Concentrations of PPX ≥10 μM produced THir cell counts that were significantly increased relative to the cultures containing only CM, whereas concentrations of 7-OH-DPAT ≥1 μM produced significant effects. A similar effect was seen when PD128,907 was studied. As was true for both PPX and 7-OH-DPAT, 3 days’ incubation with PD128,907 significantly increased THir cell counts (40%) relative to cultures containing only CM when micromolar concentrations were evaluated (F = 21.40; p < .001; Fig. 1). However, when the drug was incubated with the cultures for a longer period of time (7 days), the effect on survival was more pronounced (F = 95.37; p < .001; Fig. 1), and concentrations as low as 1 nM significantly increased THir cell counts relative to cultures containing CM. It is important to note that the magnitude of the effect observed after 7 days (168%) was primarily the result of the loss of THir cells that normally occurs in cultures in CM during 7 days because THir cell counts were significantly decreased relative to that seen after 3 days (data not shown).

None of the other DA drugs tested, including the D₃ antagonist, the D₂ agonist, and the mixed D₂/D₃ antagonists raclopride and domperidone, significantly altered the number of THir cells (Table 1). U101033E (Table 1) as well as α-tocopherol (see Fig. 5) increased the THir cell counts relative to controls. However, 10 μM U101033E was the only concentration to do so, whereas at the 100 μM concentration, all cells in the cultures died (Table 1). It is important to note that both the U101033E vehicle (Intralipid) and the 1% ethanol vehicle used with α-tocopherol were partially toxic to THir cells. Indeed, several experiments had to be discarded.

![Fig. 1. Effect of various concentrations of PPX, 7-OH-DPAT, and PD128,907 on the number of THir cells in rostral mesencephalic cultures after 3 or 7 days in culture. Values are expressed as percentage of the cell counts observed in cultures incubated with CM only. Values indicate typical response of one of the replicate experiments, and bars reflect S.D. *p < .05 versus CM.](image-url)
because the THir cell counts in the positive controls were not at least 1.75 times those seen in the PC.

**Combination Studies.** When each of the different drugs was added to cultures containing PPX and 10 μM levodopa to determine whether they altered the neuroprotective effects of PPX, only drugs with D3 activity were effective. In all experiments performed, cultures containing PPX and levodopa contained significantly more THir cells than cultures incubated with levodopa alone. In addition, 7-OH-DPAT modestly but significantly potentiated the neuroprotective effect of PPX in a dose-dependent fashion (F = 18.51; p < .001; Fig. 2). Thus, the addition of the D3-preferring agonist 7-OH-DPAT to cultures containing 100 nM PPX further enhanced the rescuing effect of PPX against levodopa-induced THir cell loss. In contrast, the D2 antagonist U99194 dose-dependently reduced the PPX (1 μM) rescuing effect (F = 10.62; p < .01; Fig. 2). Domperidone, raclopride, and the D2 agonist U95666, the only other drugs studied in these combinations studies, were without effect (Table 2). Because some of the drugs were able to alter the neuroprotective effects of PPX, they were next tested by themselves to determine whether they could alter the reduction in THir cell counts produced by levodopa.

**Neuroprotection Studies.** Only PPX (F = 23.96; p < .001) dose-dependently attenuated the THir cell loss normally produced by levodopa (Fig. 3). Levodopa (10 μM) significantly reduced the number of THir cells in culture compared with that seen with CM alone. The addition of PPX at concentrations >10 nM, however, dramatically and dose-dependently attenuated the levodopa-induced THir cell loss. When levodopa and 1 μM PPX were coincubated in the cultures, the number of THir cells was comparable to that seen in the CM control wells. The maximal rescuing effect of PPX was observed at 10 μM, where the number of THir cells was increased 253% relative to cultures incubated with levodopa alone. U101033E also increased the THir cell counts relative to cultures incubated with levodopa alone (Table 3) but only at concentrations ≥10 μM. Lower concentrations were without effect, and overall, a dose-dependent effect was not observed (F = 1.8; p = NS). α-Tocopherol also attenuated the THir cell loss induced by levodopa (F = 83.697; p < .001; see Fig. 5), but relative to cultures incubated with levodopa alone, it occurred only at the highest concentration studied (100 μM). All of the other drugs studied, including 7-OH-DPAT, U95666, U99194, domperidone (Table 3), and PD128,907 (see Fig. 6), were without effect.

The observed neuroprotective effect appeared to reflect alterations in cell survival as well as neuronal process development. Thus, in the presence of PPX and levodopa (Fig. 4b), the density of THir cells was increased relative to that seen in cultures exposed to levodopa alone (Fig. 4c). Moreover, the THir cells seen in the cultures incubated with PPX and levodopa were similar (albeit not as highly developed) to those seen in the positive control cultures (Fig. 4a), which had highly developed cell bodies with longer and more branched processes relative to those seen in cultures exposed to levodopa.

**Antioxidant Studies.** In the next series of experiments, U101033E and α-tocopherol were added to the cultures along with the D3 agonists 7-OH-DPAT and PD128,907. When fixed concentrations of U101033E and 7-OH-DPAT (1 μM), which were not by themselves neuroprotective, were incubated with mesencephalic cultures exposed to varying concentrations of levodopa, the dose-dependent THir cell loss normally produced by levodopa was attenuated (F = 45.05 p < .001; Fig. 5A). Indeed, this effect was observed at every concentration of levodopa studied except 50 μM. When various concentrations of α-tocopherol were incubated with and without a fixed concentration of 7-OH-DPAT (1 μM), the THir cell loss associated with a fixed concentration of levodopa (10 μM) was similarly attenuated. This effect overall was dependent on the concentration of α-tocopherol added.

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**Table 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>1 nM</th>
<th>10 nM</th>
<th>100 nM</th>
<th>1 μM</th>
<th>10 μM</th>
<th>100 μM</th>
<th>CM</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>U99194</td>
<td>304.5 ± 16.3</td>
<td>300 ± 5.7</td>
<td>305.5 ± 27.6</td>
<td>306 ± 7.1</td>
<td>318.5 ± 2.1</td>
<td>289.5 ± 14.9</td>
<td>302 ± 17.0</td>
<td>99.5 ± 7.8</td>
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<td>U95666</td>
<td>132.5 ± 0.7</td>
<td>143 ± 17.0</td>
<td>139.5 ± 0.7</td>
<td>140 ± 19.8</td>
<td>135 ± 35.4</td>
<td>145.5 ± 17.7</td>
<td>148.5 ± 16.3</td>
<td>14 ± 5.7</td>
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<tr>
<td>Raclopride</td>
<td>136 ± 19.8</td>
<td>144.5 ± 13.4</td>
<td>132 ± 21.2</td>
<td>142.5 ± 9.2</td>
<td>141.5 ± 17.7</td>
<td>131.5 ± 12.0</td>
<td>148.5 ± 16.2</td>
<td>14.2 ± 5.6</td>
</tr>
<tr>
<td>Domperidone</td>
<td>278 ± 30.0</td>
<td>322 ± 58.0</td>
<td>306.5 ± 7.83</td>
<td>304 ± 52.3</td>
<td>306.5 ± 24.7</td>
<td>257 ± 31.1</td>
<td>290.5 ± 9.2</td>
<td>110.5 ± 24.7</td>
</tr>
<tr>
<td>U101033E</td>
<td>Not done</td>
<td>97 ± 2.8</td>
<td>97 ± 4.2</td>
<td>117 ± 8.5</td>
<td>464 ± 56.6*</td>
<td>100.5 ± 7.8</td>
<td>32 ± 9.9</td>
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</tr>
</tbody>
</table>

*p < .05.
versus levodopa only. A similar result was observed when PD128,907 was alone (compare Table 3 [7-OH-DPAT results] and data in Fig. 5B). Moreover, the combined effects of α-tocopherol and 7-OH-DPAT appeared to be synergistic as evidenced by the significant interaction term ($F = 50.65, p < .001$; Fig. 8). Again, recipient cultures incubated with the uncut conditioned media incubated without drug (DM) had significantly more THir cells than that seen in PC. In addition, the >10-kDa fractions dose dependently increased THir cell counts in recipient cultures. Because the conditioned media was more concentrated as a result of the fractionation, its effect on THir cell counts was significantly higher than that seen in cultures incubated in CM containing FCS. THir cell counts in cultures incubated with the <10-kDa fractions were similar to those seen in the PC cultures (data not shown).

### Discussion

In all experiments, THir cell counts were used as the dependent measure for DA neurons. THir cell counts are highly correlated with the number of cells immunoreactive for other markers of DA neurons (Ptak et al., 1995) due to the dissection procedure (To-mozawa and Appel, 1986). It is therefore assumed that the THir cell counts reported here reflect DA neurons. The number of THir cells in culture decreases naturally (Brundin and Bjorklund, 1987), which was confirmed in the present study because the number of THir cells in the plate and positive controls from the PD128,907 study (Fig. 1) were significantly reduced after 7 days in culture relative to counts seen after 3 days. The neuroprotective effects seen in the present study after D₃ agonist incubation can therefore be described in terms of increased survival or a rescuing effect. However, because “toxicity” measures were not gathered in these studies and it is known that the phenotypic expression of TH can be turned on and off (Bowenkamp et al., 1996), the neuroprotective effects observed here are best characterized as a rescue, increased survival, and/or maintenance of the expression of the TH phenotype.
Two lines of evidence from the present results are consistent with the hypothesis that the PPX D3 actions are involved in its neuroprotective effects. First, among all the drugs evaluated in the "survival" studies (experimental series 1), only the D3 agonists 7-OH-DPAT and PD128,907 increased THir cell counts in a dose-dependent fashion similar to that seen with PPX. Second, among all the drugs evaluated in the "combination" studies (experimental series 2), the D3 agonist 7-OH-DPAT potentiated and the D3 antagonist U99194 attenuated the neuroprotective effect PPX had against levodopa-induced THir cell loss. None of the other drugs studied were effective in this regard. Although these findings favor the involvement of D3 in the neuroprotective effect of PPX, issues involving receptor specificity as well as concentration dependence should be considered.

Binding studies reveal that among DA agonists, PPX, PD128,907, and 7-OH-DPAT are considered to be the most specific for D3 receptors (Levant, 1997). When compared directly at the agonist binding site, the affinities of PPX and 7-OH-DPAT for D3 receptors are only approximately 5- to 7-fold higher than that seen for D2 receptors (Piercey et al., 1996b). PD128,907 exhibits similar affinities using functional assays (Levant, 1997). Because these affinity ratios are not greater than the several hundred-fold normally required

### TABLE 3

<table>
<thead>
<tr>
<th>Drug</th>
<th>Levodopa Alone</th>
<th>1 nM</th>
<th>10 nM</th>
<th>100 nM</th>
<th>1 μM</th>
<th>10 μM</th>
<th>100 μM</th>
<th>CM</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-OH-DPAT</td>
<td>53 ± 15.6</td>
<td>54.5 ± 16.3</td>
<td>60.5 ± 9.2</td>
<td>56.5 ± 4.9</td>
<td>52 ± 5.7</td>
<td>46.5 ± 0.7</td>
<td>50 ± 2.8</td>
<td>294 ± 18.4</td>
<td>84.5 ± 9.2</td>
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<tr>
<td>U95666</td>
<td>82.5 ± 6.4</td>
<td>67 ± 11.3</td>
<td>79 ± 12.7</td>
<td>77 ± 18.4</td>
<td>70 ± 4.2</td>
<td>70 ± 4.2</td>
<td>23.5 ± 7.8</td>
<td>305.5 ± 10.6</td>
<td>98.5 ± 10.6</td>
</tr>
<tr>
<td>U99194</td>
<td>66.5 ± 14.8</td>
<td>68.5 ± 17.7</td>
<td>69 ± 8.5</td>
<td>79.5 ± 13.4</td>
<td>74 ± 4.2</td>
<td>73.5 ± 9.2</td>
<td>67 ± 2.8</td>
<td>302 ± 99.5</td>
<td>99.5 ± 7.8</td>
</tr>
<tr>
<td>Domperidone</td>
<td>55 ± 15.6</td>
<td>58.5 ± 23.3</td>
<td>46.5 ± 12.0</td>
<td>57 ± 8.5</td>
<td>64.5 ± 29.0</td>
<td>65.5 ± 13.4</td>
<td>51.5 ± 24.7</td>
<td>290.5 ± 9.2</td>
<td>110.5 ± 24.7</td>
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<tr>
<td>U101033E</td>
<td>43 ± 4.2</td>
<td>Not done</td>
<td>47 ± 5.6</td>
<td>50 ± 11.3</td>
<td>46.5 ± 11.3</td>
<td>94.5 ± 13.4</td>
<td>226 ± 2.8*</td>
<td>200.5 ± 7.8</td>
<td>52 ± 9.8</td>
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</table>

All drug concentrations are in addition to 10 μM levodopa. *p < .05 versus levodopa.

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**Fig. 4.** Photomicrographs of THir cells in rostral mesencephalic cultures after 72-h incubation with CM containing FCS (a), CM with 1 μM pramipexole and 10 μM levodopa (b), or CM and 10 μM levodopa (c). Bar in a applies to b and c also (bar, 20 μm).

**Fig. 5.** Effects of U101033E (A) and α-tocopherol (B) on THir cell counts in mesencephalic cultures after 72 h. A, various concentrations of levodopa were added to the cultures alone or with 1 μM U101033E and 1 μM 7-OH-DPAT. Values are expressed as percentage of the cell counts observed in cultures incubated with CM only. B, various concentrations of α-tocopherol were added with a fixed concentration of levodopa (10 μM) or with a fixed concentration of levodopa (10 μM) and a fixed concentration of 7-OH-DPAT (1 μM). Values indicate typical response of one of the replicate experiments, and bars reflect S.D. 0, levodopa only; *p < .05 versus levodopa only.
to qualify a drug as being receptor subtype specific, the term “D₃-preferring” has been used to describe this drug group. However, because of the broad dose-response curves used in the present study and the fact that PPX significantly increased THir cell counts in the survival studies only at the 10 μM concentration where significant D₂ activity would be anticipated, it could be argued that D₃ receptor activity is not primarily responsible. Although this is a valid argument, we do not believe that it applies in this case. First, in the 72-h survival studies, 7-OH-DPAT and PD128,907 produced significant effects at 1 μM. However, when the effects of PD128,907 on survival were studied over a longer period (7 days), concentrations as low as 1 nM were shown to significantly reduce THir cell loss. Second, if the D₂ actions of PPX were responsible for the neuroprotective effect, then U95666, which possesses 1000-fold greater affinity for D₂ than D₃ receptors (R. Huff, unpublished observation), should have increased the THir cell counts in the survival studies, which was not the case. Third, U95666 was unable to potentiate the neuroprotective effect of PPX in the combination studies, whereas 7-OH-DPAT was effective. Although the enhanced neuroprotective benefit seen with 7-OH-DPAT was modest at best, it may have been possible to demonstrate more pronounced effects had more extensive dose-response studies been performed for longer periods of time. Regardless, because the D₃ antagonist U99194, which has been shown to possess 20- to 30-fold (Piercey et al., 1996b) greater affinity for D₂ than D₃ receptors (R. Huff, unpublished observation), should have increased the THir cell counts in the survival studies, which was not the case. The involvement of D₃ is strongly suggested. Again, however, why domperidone, and especially the less D₂-preferring antagonist raclopride (Sokoloff et al., 1990; Levant, 1997), did not exhibit effective attenuation at higher concentrations where D₃ blockade would be expected remains unknown. Taken together, these studies support a role for the D₃ receptor in the neuroprotective effects of PPX more so than its D₂ actions. This interpretation would, of course, depend on

Fig. 6. Effect of various concentrations of levodopa, levodopa with (W) 100 nM PD128,907, or levodopa with 100 nM PD128,907 and 100 nM α-tocopherol on THir cell counts in rostral mesencephalic cultures after 72 h. Values are expressed as percentage of the cell counts observed in cultures incubated with CM only. Values indicate typical response of one of the replicate experiments, and bars reflect S.D. *p < .05 versus the levodopa value at that concentration.

Fig. 7. Effect of DM conditioned by exposure to various concentrations of PD128,907 for 7 days on the THir cell counts in freshly harvested rostral mesencephalic recipient cultures after 72 h. Values indicate typical response of one of the replicate experiments, and bars reflect S.D. 0, recipient cultures incubated with fresh defined media and the drug vehicle (Hanks’ balanced salt solution). CM, recipient cultures incubated with fresh CM containing 10% FCS. *p < .05 versus 0 (DM); t p < .05 versus PC.

Fig. 8. Effect of >10-kDs fractions of media from donor cultures conditioned by exposure to 10 and 100 μM PD128,907 for 6 days on THir cell counts in recipient rostral mesencephalic cultures. Values indicate typical response of one of the replicate experiments, and bars reflect S.D. DM, recipient cultures incubated with media from donor cultures containing DM without PD128,907. PC, PCs that were recipient cultures incubated in fresh DM and the drug vehicle (Hanks’ balanced salt solution). CM, recipient cultures incubated with fresh CM containing 10% FCS. *p < .05 versus PC.
the presence of D₃ receptors on the THir cells in culture. That D₃ receptors are present on DA neurons is supported by the fact that D₃ mRNA and receptor protein have been detected in the substantia nigra and ventral tegmental area in most studies and that lesions of the midbrain dopaminergic system reduce D₃ receptor density in the nucleus accumbens and D₃ mRNA in the substantia nigra (for a discussion and the controversies surrounding the detection of D₃ receptor, see Sokoloff et al., 1990; Valerio et al., 1994; Diaz et al., 1995; Griffon et al., 1995, Levesque et al., 1995; Le Moine and Bloch, 1996; Tepper et al., 1997). Interestingly, the D₃ receptor has been implicated in DA neuron development because D₃ receptor activation was shown to increase branching and neurite extension in both MN9D cells and in mesencephalic cultures (Swarzenski et al., 1994), suggesting that D₃ receptors are not only present but also functional in culture.

If D₃ receptor activation was solely responsible for the neuroprotective effects of PPX, then 7-OH-DPAT and PD128,907 should have attenuated the THir cell loss produced by levodopa when these drugs were added to the cultures alone. This was not the case, however. Even when high micromolar concentrations of these drugs were evaluated in the levodopa toxicity model, they did not attenuate the loss of THir cells. Actions other than D₃ receptor affinity may explain this effect. Direct comparisons of PPX and 7-OH-DPAT reveal few differences in their affinities for DA, noradrenergic, cholinergic, and serotonergic receptors (Piercey et al., 1996b). Mechanisms in addition to D₃ receptor agonism must therefore be involved.

We previously demonstrated that the inactive stereoisomer of PPX was partially neuroprotective against the effects of levodopa, suggesting that the antioxidant effects of PPX also contributed to its neuroprotective action (Carvey et al., 1997a). The results from the present study strongly support that hypothesis. Thus, the addition of the antioxidants U101033E or α-tocopherol and 7-OH-DPAT at concentrations that were not neuroprotective attenuated the THir cell loss when added in combination. In addition, an apparent synergism was observed when 10 μM α-tocopherol was added because even at this concentration, α-tocopherol was not shown to provide any significant neuroprotective benefit, whereas in combination with 7-OH-DPAT, a 300% increase in THir cell counts was observed. Because of the partial toxicity associated with the vehicles for the U101033E (1% Intralipid) and α-tocopherol (1% ethanol), it is possible that an unknown interaction could have been responsible for this effect. However, this did not appear to be the case because a similar effect was observed when 100 nM PD128,907 and 100 nM α-tocopherol were combined. In a sense, by adding antioxidants along with a D₃-preferring agonist with receptor binding properties similar to those of PPX, the primary pharmacological actions of PPX were mimicked and shown to be neuroprotective. This suggests that the combined actions of PPX as a D₃-preferring agonist and antioxidant are responsible for its neuroprotective benefit. The need for both of these properties might also explain why U99194 was only partially effective at attenuating the neuroprotective effects of PPX, whereas domperidone and raclopride were without effect. Thus, these drug combinations would block the D₃ or D₂ actions of PPX but not its antioxidant actions.

Because oxidant stress is thought to be responsible for the loss of THir cells in mesencephalic cultures exposed to levodopa, the antioxidant properties of PPX might be primarily responsible for its neuroprotective benefit. However, only very high concentrations of the antioxidants were shown to be effective in all of the assays in which they were evaluated. These micromolar concentrations needed for neuroprotection by α-tocopherol are similar to those observed previously (Gabby et al., 1996). It thus seems more likely that a combined action involving D₃ receptor activation and antioxidant action is responsible for the neuroprotective effect of PPX.

The consequence of D₃ receptor activation may involve increased production of a DA neuron autotrophic activity, which in turn provides neuroprotective benefit. It has been previously hypothesized that such a factor exists (Dal Toso et al., 1988), and the results from the present study as well as previous studies in our laboratory support this contention. Thus, in the present study, recipient culture THir cell counts in wells containing conditioned media from control donor cultures were significantly higher than the counts seen in recipient cultures incubated with DM only. Because the donor cultures also contained DM, the increase in THir cell counts observed must have been the result of some product of the donor mesencephalic cultures. Moreover, this activity was increased by all of the D₃-preferring agonists but not by the other drugs studied. We have previously shown that the effect produced by this conditioned media is heat labile and not seen in parietal cortex cultures or mesencephalic cultures pretreated with the DA neurotoxin MPP+, suggesting the involvement of a protein product of a DA neuron (Carvey et al., 1997b; Ling et al., 1998b). In addition, a preliminary characterization of this activity suggested the involvement of a 35-kDa protein, which is consistent with the results reported here (Ling et al., 1998b). Thus, in the final experiment, the conditioned media was separated into a <10-kDa and a >10-kDa fraction to eliminate the possibility that PD128,907 (which would be in the <10-kDa fraction) carried over in the conditioned media was responsible for the increase in THir cells. Activity was present in only the >10-kDa fractions. Because this effect could not have been a consequence of drug carry over, it is likely the result of an increase in PD128,907-induced trophic activity.

Because all of the D₃ agonists increased the conditioned media transfer effect, it would be anticipated that they should all be neuroprotective in the levodopa toxicity model. The fact that only PPX was effective in this regard may suggest that the increase of protein or proteins produced by the D₃ agonists are oxidant labile. Thus, either the increase in trophic activity produced by 7-OH-DPAT and PD128,907 was insufficient to enhance the growth and/or survival of THir cells, or the trophic activity was increased but then degraded by the free radicals produced by levodopa. The latter hypothesis seems most applicable because both 7-OH-DPAT and PD128,907 were able to increase THir cell counts in the survival studies, suggesting that the trophic activity produced was able to enhance THir survival. However, if the protein itself was oxidant labile, the free radical oxidative environment present in cultures incubated with levodopa would reduce the activity of the trophic molecule precluding a trophic action. In the presence of free radical scavengers such as α-tocopherol, U101033E, or that inherent in PPX itself, the degree of nucleophilic attack on the protein would be reduced. Because other manipulations of the DA receptor
were not shown to be neuroprotective and only D₃ receptor agonists were shown to enhance trophic activity, it is concluded that the increased trophic activity is involved in the neuroprotective effect seen with PPX.

In summary, the results from the present study are consistent with prior studies from our laboratory as well as others demonstrating a neuroprotective benefit of PPX in several in vitro and in vivo models. Based on the results presented here, it appears that this neuroprotective effect is a consequence of the combined actions of PPX as a D₃ agonist and its antioxidant properties. Because PPX is also a potent D₃ agonist, which is most likely responsible for its efficacy in the treatment of PD, its added action as a DA neuroprotectant may slow the rate of DA neuron loss in patients. This hypothesis is currently being tested in a prospective study, although the very high compliance rate seen in patients on PPX monotherapy after 42 months (84%; Carion et al., 1998) suggests a reduced need for levodopa treatment, which is not inconsistent with a neuroprotective benefit.

References


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